Relationship of Aberrant DNA Methylation in Inheritable and Sporadic Endometrial Cancer Carcinogenesis

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Contents

Abstract

DNA methylation is essential for healthy mammalian development and function, and is involved in important processes such as suppression of repetitive elements, genomic imprinting and carcinogenesis. Thus, aberrant DNA methylation is linked to some of the adverse outcomes. DNA methylation indicates addition of a methyl (CH_3) group to fifth position of a cytosine within CpG dinucleotides, which forms clusters called CpG islands. DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs). Inactivation of certain tumor suppressor genes caused by methylation of the promoter region is widely observed in various types of cancer.

Epigenetic reprogramming, including DNA demethylation, occurs in mammalian primordial germ cells (PGCs) and early embryos, and returns the cells to pluripotency. Therefore, DNA methylation in human cells has long been thought not to be inherited. However, recent studies have raised the possibility that epimutation can occur in human cells.

In chapter 1, I focus on Lynch syndrome, an inherited cancer syndrome that is caused by germline mutation of DNA mismatch repair (MMR) genes and has an increased risk of colorectal, endometrial and other cancers. Recent studies have shown that 25-30% of patients with Lynch syndrome have no germline mutation of MMR genes. This raises the possibility that epimutation of MMR genes could be an alternative cause of Lynch syndrome. Therefore, I investigated epimutation of MMR genes in peripheral blood DNA in 106 patients with endometrial cancer, and identified patients with Lynch syndrome.

Epimutation could be a cause of inherited cancer syndrome and sporadic cancer. Some types of cancers show concomitant DNA methylation of multiple genes, which is referred to as CpG island methylator phenotype (CIMP). In colon cancer, Lynch syndrome and CIMP-positive cancer show similar clinicopathological features. However, there are few studies on CIMP-positive endometrial cancer, and the causes and features of this condition are unknown. In chapter 2, I hypothesized that patients with

CIMP-positive endometrial cancer have aberrant DNA methylation (epimutation) in normal tissue that is a trigger of carcinogenesis. Therefore, I investigated the genome-wide methylation status of DNA from peripheral blood cells (PBCs) and cancer tissue in patients with CIMP-positive and CIMP-negative endometrial cancer. In DNA from PBCs, the promoter region of miR-663a was significantly hypermethylated in CIMP-positive cases compared to CIMP-negative cases.

These studies provide new knowledge on the relationship between epigenetic abnormalities and endometrial carcinogenesis. The findings may be applicable to early detection and as a predictive marker. Furthermore, DNA methylation is reversible, and future strategies for DNA demethylation may contribute to cancer prevention.

Abbreviations

Atypical endometrial hyperplasia: AEH

CIMP-high: CIMP-H

CIMP-low: CIMP-L

CIMP-negative: CIMP(-)

CpG island methylator phenotype: CIMP

Differentially methylated CpGs: DMCs

Differentially methylated regions: DMRs

DNA methyltransferase: DNMT

DNA mismatch repair gene: MMR gene

Methylation specific polymerase chain reaction: MSP

MicroRNAs: miRNAs

Next-generation sequencing: NGS

Peripheral blood cells: PBCs

Polymerase chain reaction: PCR

Post-Bisulfite Adaptor Tagging: PBAT

Primordial germ cells: PGCs

Transcription start sites: TSS

General Introduction

DNA methylation is one of the epigenetic mechanisms used to regulate gene expression. Among several mechanisms regulating gene expression, DNA methylation is the most common for fixing genes in the "off" position. Thus, DNA methylation plays important roles in embryonic development, chromosome stability and carcinogenesis. Indeed, the relationship between methylation abnormalities and human diseases such as cancer, psychiatric disorder and congenital imprinting disorders are currently being studied (1). The results of these studies will be important for not only treatment of these diseases but also understanding of DNA methylation mechanisms and prevention of DNA methylation abnormalities.

DNA methylation occurs by the addition of methyl groups to cytosine bases in mammalian DNA by DNMTs. In mammals, there are 3 major DNMTs: DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b are de novo DNMTs that show equal affinity for hemi-methylated DNA (DNA with one strand methylated) and non-methylated DNA (2). In contrast,

DNMT1 is a maintenance DNMT that binds to hemi-methylated DNA at CpG sites. After DNA replication, the parent strand remains methylated, but the daughter strand is not methylated. DNMT1 binds to these hemi-methylated CpGs and methylates the cytosine on the newly synthesized daughter strand, and maintains CpG methylation patterns through mitosis (3).

Unlike animals, plants do not have a separate germline in which epigenetic marks are erased and reestablished. Thus, even if DNA methylation machinery is restored, epigenetic changes induced in DNA methylation abnormalities can be maintained and inherited (4). One of the oldest examples of heritable epigenetic change (epimutation) in plants is a morphological defect in the development of flower in *Linaria vulgaris*. The mutant phenotype is due to aberrant DNA methylation and transcriptional suppression of *Lcyc*, which is a regulator of dorsoventral asymmetry (Figure 1). In correlation with the expression recovery by demethylation of *Lcyc* gene, phenotype is restored occasionally (5).

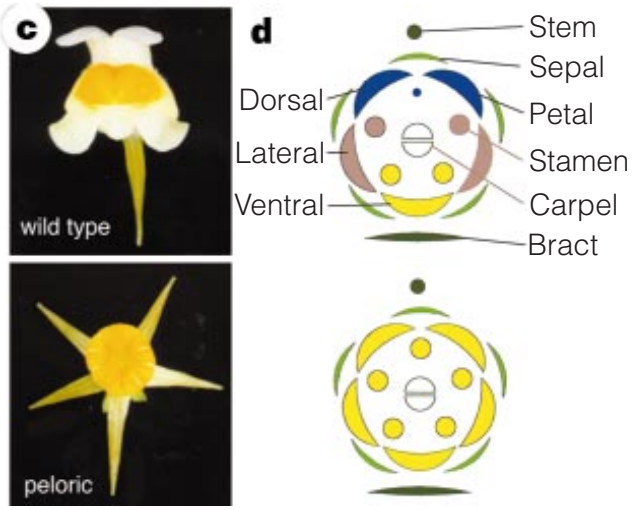

Figure 1 Wild-type and peloric *Linaria vulgaris* flowers.
c, Face view of a wild-type *Linaria* flower compared to a peloric mutant.
d, Floral diagrams of wild-type (top) and peloric (bottom) flowers showing the relative positions of different organs.
Adapted from Cubas, P. et al. :Nature, 401: 157-161, 1999

In contrast, methylation patterns of mammalian cells are erased in PGCs and at the post-fertilization stage (Figure 2). Therefore, epimutation was thought not to occur in mammals. However, there is increasing evidence that environmental (nutritional) stimuli can modify DNA methylation and affect phenotypic expression of genes. For example, the methylation level of the *Leptin* promoter is significantly increased in oocytes of high-fat diet mice. Female offspring from the obese mice showed higher methylation level of

Leptin promoter in the liver than normal mice. Expression level of *Leptin* was also significantly decreased in the liver of these offspring (6). Examples of epigenetic inheritance in mice raise possibility that occurs similar event in humans.

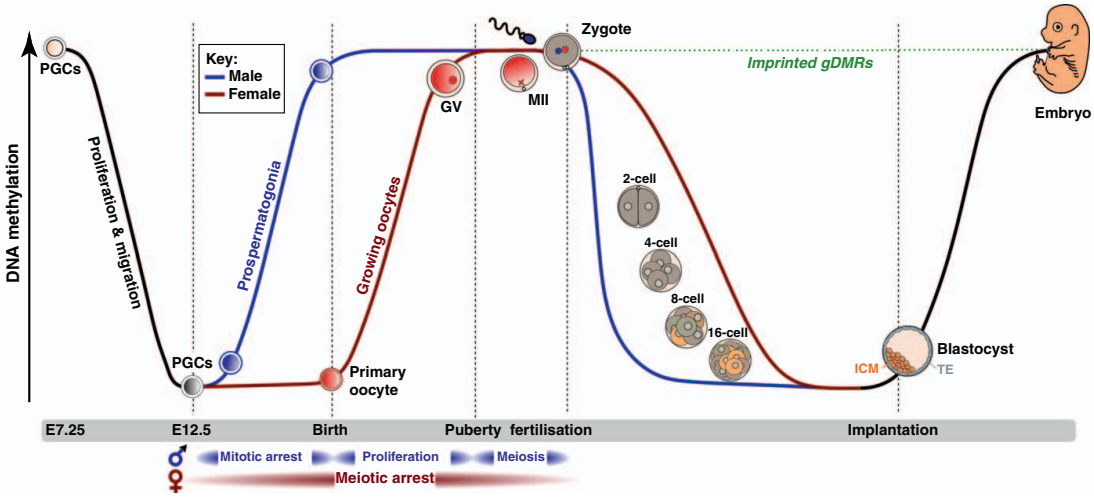

Figure 2. DNA methylation changes during developmental epigenetic reprogramming.
Adapted from Smallwood, SA. et al. : Trands Genet, 28: 33-42, 2012

To examine this issue further, I focused on Lynch syndrome, which is an inherited cancer syndrome caused by germline mutation of DNA

mismatch repair (MMR) genes, most frequently *MLH1* and *MSH2* (7, 8). Lynch syndrome is characterized by increased risk of colorectal, endometrial, ovarian and other cancers. In Knudson's two-hit theory, it is required that an abnormality (hit) occurs in both alleles of a tumor-suppressor gene for disease progression (Figure 3). Germline mutations generally represent the

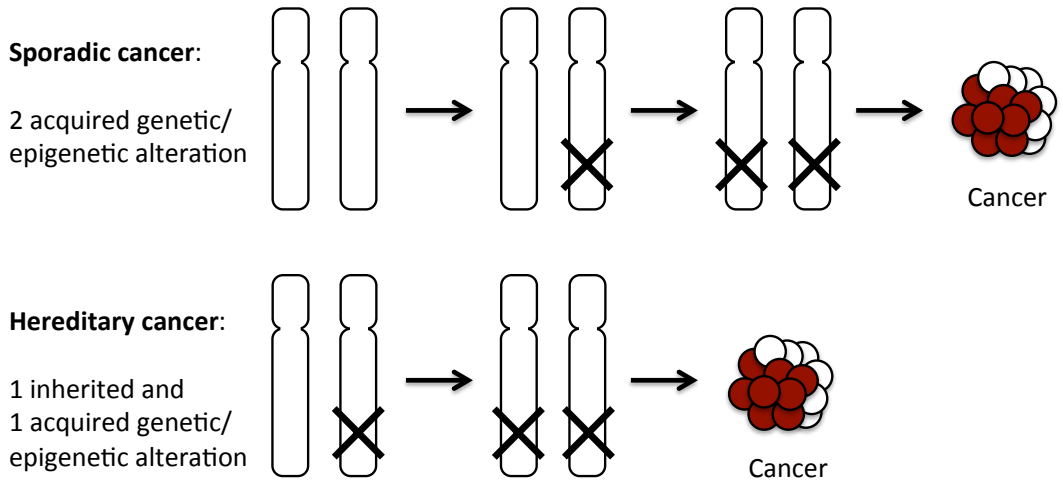

Figure 3. Knudson's two-hit theory
Modified from http://ocw.tufts.edu/data/20/300759/300840_xlarge.jpg

first hit on one allele, while the second hit typically results from a sporadic mutation, loss of heterozygosity or methylation. However, germline

mutations of MMR genes are not found in 25-30% of patients with Lynch syndrome (9-12). Therefore, it is possible that epimutation of MMR genes acts as the first hit in patients with no germline mutation.

Evaluation of epimutation of MMR genes to date has been based on case studies with insufficient information on families, and epimutation in families with endometrial cancer has not been examined. In chapter 1, I explored epimutation in cases of endometrial cancer and identified patients with Lynch syndrome.

Epimutation could be a cause of inherited cancer syndrome and sporadic cancer. Aberrant DNA hypermethylation in CpG island is a hallmark of cancer and is characterized by tumor-specific hypermethylation of numerous CpG islands (13). *MLH1* methylation is also observed in cases of sporadic colorectal and endometrial cancer (14). These cancers show the same phenotype of mismatch repair defect and clinicopathologic characteristics similar to Lynch syndrome. Such sporadic colorectal cancer also has a close relationship with cancer with a CpG island methylator

phenotype (CIMP) (15) (Figure 4). CIMP was first proposed by Toyota et al. in 1999 (16). They defined a subgroup of colorectal cancers with concurrent

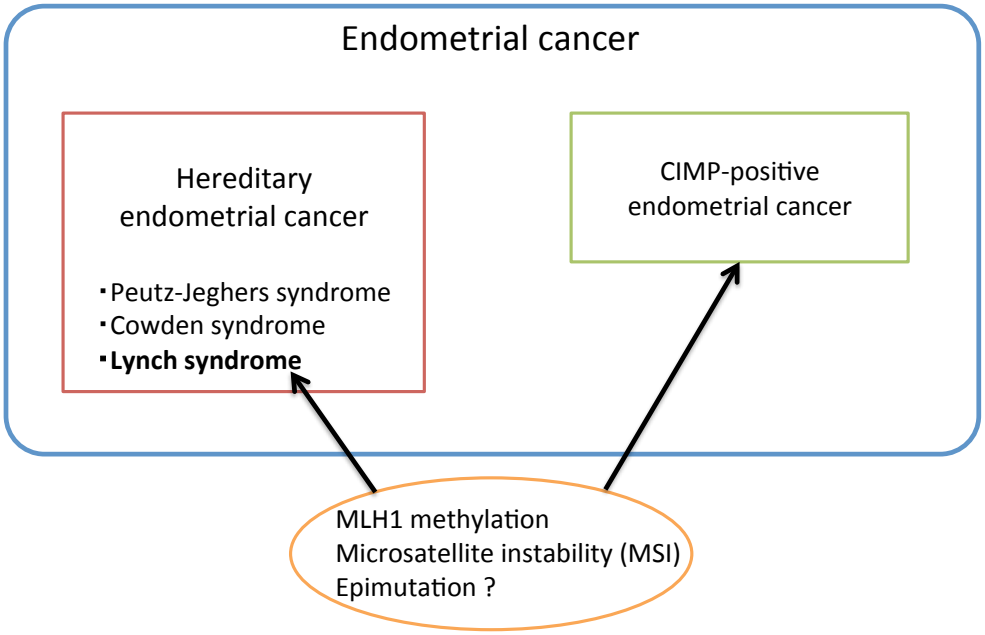

Figure 4. Relationship between Lynch syndrome and CIMP-positive endometrial cancer

multiple promoter hypermethylation of tumor-related genes as CIMP. CIMP has subsequently been reported in gastric (17, 18), lung (19, 20), liver (21) and ovarian (22) cancer. Recent studies revealed that CIMP is negatively associated with genetic mutations in colorectal cancers (23, 24),

which suggests that it can provide an alternative oncogenic pathway. This findings supported that epimutation may be one of the cause of CIMP positive cancer. Thus, I hypothesized that normal tissue of CIMP-positive endometrial cancer has features which are prone to get DNA methylation, and that such features play important roles in carcinogenesis in this cancer.

General Discussion

Lynch syndrome is one of the most prevalent hereditary cancer syndromes in humans and is caused by inherited defects in MMR genes (89, 90). In the last two decades, increased appreciation of epigenetic mechanisms in tumorigenesis and identification of constitutional epimutations underlying Lynch syndrome have laid the foundation for the epigenetic era (11). Epimutation is regarded as secondary if induced by an adjacent genetic alteration, and otherwise as primary (33). Lynch syndrome offers one of the first examples of cancer-associated constitutional epimutation, namely primary epimutation of *MLH1* (11). Recent observations of constitutional epimutations as the first hit and promoter methylation as the second hit in Lynch syndrome emphasize the increasing significance of epigenetic events, especially as methylation as the second hit is associated with a more generalized CIMP in tumors (91).

CIMP was first explained by Toyota et al. in 1999 (16). CIMP occurs in a subset of colorectal cancers that are characterized by vast

hypermethylation of promoter CpG island sites, resulting in inactivation of several tumor suppressor genes or other tumor-related genes (92). Many studies have found an association between CIMP status and other important epidemiological and molecular factors, such as smoking, age and genetic mutations (93-95).

In chapter 1, I tried to identify MMR genes epimutation positive patients from 106 endometrial cancer patients. According to our preliminary experiment, 1% of endometrial cancer patients show *MLH1* epimutation, but no cases of epimutation-positive endometrial cancer were found in this study.

I also identified two patients with Lynch syndrome among the 106 patients with endometrial cancer, based on the Amsterdam II criteria and revised Bethesda guidelines (diagnostic criteria for Lynch syndrome using family and personal history). One of these patients had a novel *MSH6* nonsense mutation. Since colon cancer in patients with Lynch syndrome is characterized by mutations in *MLH1* and *MSH2*, rather than *MSH6*,

endometrial cancer with Lynch syndrome may have a different carcinogenetic pathway.

Next, I focused on the relationship between epigenetic alteration and sporadic endometrial carcinogenesis. Many studies have shown that DNA methylation is related to endometrial cancer, especially in the early stages of carcinogenesis. Moreover, some types of cancer, including endometrial cancer, show a CIMP phenotype. These findings suggest that DNA methylation plays important roles in carcinogenesis and in cancer phenotypes, but it is still unclear whether such DNA methylation is a cause or a result of cancer.

I succeeded in identifying aberrant DNA methylation, which is a potential cause of CIMP-positive endometrial carcinogenesis. The MiR-663a promoter region in PBC DNA of CIMP-H patients was significantly hypermethylated compared to that of CIMP(-) patients. Consistent with this methylation pattern, miR-663a expression in PBCs of CIMP-H patients was lower than that in CIMP(-) patients. The miR-663a promoter region is a

hypomethylated region in normal human adult tissues, and therefore miR-663a DNA methylation identified in this study may be a novel epimutation candidate.

This study provides new findings on the involvement of epigenetic abnormalities in hereditary and sporadic endometrial cancer. Detection of abnormal DNA methylation using a blood specimen can be performed quickly and conveniently, and thus is useful as a cancer prediction and diagnostic marker. Furthermore, since methylation of DNA is reversible, site-specific demethylation may contribute to prevention of cancer.

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